PLASTIN PROMOTER DIRECTED GENE THERAPY

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application 60/110,844, filed December 4, 1998, which is herein incorporated by reference in its entirety. This application is also related to U.S. Disclosure Document 441607, filed August 7, 1998, which is also herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates generally to the treatment of cancer using plastin promoter directed gene therapy.

BACKGROUND OF THE INVENTION

Gene therapy is one of the newest approaches to cancer treatment, in which the transfer of genetic material into cells alters their phenotype in a beneficial manner. Adenoviral vectors are currently the most commonly used vector for cancer gene therapy because of their high titers, ease of production, high infection efficiency for epithelial neoplastic cells, and the fact that their transcriptional units are expressed extrachromosomally in non dividing cells. The broad host range of this virus also results in the infection of both the intended tumor cells as well as of the normal surrounding tissue. This limits the utility of the vectors, especially when the vector gene products relate to sensitization of the tumor cells to chemotherapy or radiation therapy, due to toxicity to the normal cells (Descamp *e.g.*, 1996; Bramson *et al.*, 1995; Zhang *et al.*, 1994).

One way to circumvent this limitation is to use a tissue-specific transcriptional promoter which is active only in the target tumor cells. A number of promoter/enhancer sequences have been characterized for application in gene therapy methods, potentially allowing for the tightly regulated expression of therapeutic genes within neoplastic tissues (Patterson *et al.*, 1999). For instance, in breast cancer, transcriptionally targeted gene expression can be achieved using breast-tissue-specific or disease-specific promoters or, alternatively, through exploiting promoter elements that are responsive to conditions unique to solid tumor microenvironments. Few such promoter elements have been

identified to date. Accordingly, the need for the identification and development of tightly regulated promoters for the delivery of agents to neoplastic tissues remains great.

SUMMARY OF THE INVENTION

The present invention is based on the development of vectors in which a plastin promotor is used to effect the expression of therapeutic transgenes in neoplastic but not in normal epithelial cells.

The invention includes *in vivo* delivery vectors comprising a plastin promoter operably linked to a gene whose induction modifies the metabolism of a cell. Preferred vectors include a human plastin promoter, particularly a L-plastin promoter. Vectors of the invention may be replication deficient, replication competent or conditionally replication competent. Vectors of the invention may be contained within a recombinant adenovirus and may be formulated into pharmaceutical compositions.

The gene encoded by the vectors of the invention include genes whose induction modifies the metabolism of a cell. Such genes include, but are not limited to, a thymidine kinase, cytosine deaminase, a purine nucleotide phosphorylase, a nitroreductase, β-galactosidase, a cytochrome P450, a deoxycytidine kinase and a thymidine phosphorylase.

The invention further includes methods of sensitizing tumor cells to a chemotherapeutic agent, comprising the steps of infecting at least a fraction of the tumor cells with a vector of the invention and administering a prodrug.

The invention also includes methods of removing cancer cells from bone marrow or peripheral blood mononuclear cells comprising the steps of infecting at least a fraction of the tumor cells with a vector of the invention and administering a prodrug. Such prodrugs include, but are not limited to 6-methoxypurine arabinonucleoside, acyclovir, ganciclovir and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil, 6-methylpurine-2'-deoxyribonucleotide, 5-fluorocytosine, a dinitrobenzamide mustard derivative, cyclophosphamide, ifosfamide, 1-β-D-arabinofuranosylcytosine and 5'-deoxy-5-fluorouridine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Light Microscopic Study of the LacZ gene expression levels in different cell lines. 1.4×10^5 cells were infected at 0, 5, 20, and 80 MOI with Ad-LP-LacZ or Ad-CMV-LacZ virus vector for 90 minutes. After infection, the cells were incubated in duplicate 6 well plates for 48 hours, after which X-Gal staining was performed. The positive cells were counted in the high powered field of the light microscope. A: CCD cell line, Ad-CMV-LacZ at 20 MOI, 200X; B: EJ cell line, Ad-CMV-LacZ at 20MOI, 200X; C: CCD cell line, Ad-LP-LacZ at 80MOI, 100X; D: EJ cell line Ad-LP-LacZ at 80MOI , 100X.

Figure 2. Time Course of Ad-CMV-LacZ and Ad-LP-LacZ in different cell lines. 1.4×10^5 cells were infected at different MOI of the Ad-CMV-LacZ and Ad-LP-LacZ vectors. After infection, cells were seeded in 6 well plates in duplicate. X-Gal staining was performed at day 2,4,5,6,7 and the positive cells were counted in the high power field of a light microscope. The expression of LacZ gene reached a maximum in the cells infected by the Ad-CMV-LacZ at day 2, and maintained a longer time as the vector MOI was increased. The accumulation of the gene product in Ad-LP-LacZ is slow but finally reached the maximum expression at day 4.

Figure 3A-3B. Toxicity of Vectors Figure 3A: The different cell lines were infected at varying MOI (20, 80, 160) using the Ad-CMV-CD or Ad-LP-CD virus vectors. The infected cells and non-infected cells were mixed in varying ratios to generate 0, 5%, 20%, 30%, 40%, 50%, 60%, 100% percentages of infected cells. Cells were then seeded in 6 well plates and incubated for 5 days in 500μmol 5-FC. The cells were then trypsinized and surviving cells were counted by Trypan Blue exclusion. Figure 3B: Tumor Cell killing efficiency with Ad-CMV-CD and Ad-Lp-CD vectors. 1.4×10^5 cells were infected at 0, 5 and 20 MOI with the Ad-CMV-CD or Ad-LP-CD vectors for 90 minutes, washed in PBS and then seeded in the 6 well plates in duplicate. Cells were then incubated with 500μmol 5FC for 5 days and stained with 0.5% Methylene blue for 30 minutes. Surviving cells stayed in the plates. (Up: Nutu-19 cell line; below : EJ cell line). In Nutu-19 cell line, 5MOI of Ad-CMV-CD killed almost all the cells and 20MOI of Ad-LP-CD can kill

all the cells. In EJ cell line, 5MOI of Ad-CMV-CD killed all the tumor cells and 5MOI of AD-LP-CD can kill about 95% of the tumor cells.

Figure 4. Study of the toxicity of the backbone vs. CD Vector. $1.4x10^5$ cells were infected at 0, 5, 20, 40,80 and 160 MOI vector with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD or Ad-Lp-CD vectors for 90 minutes and seeded in 6 well plates in duplicate. Cells were then incubated in 500μmol 5FC for 5 days. The percentage of surviving cells was counted by trypan blue exclusion. No toxicity was seen at a 80MOI with the Ad-CMV-LacZor Ad-Lp-LacZ vectors. In contrast, 100% of Nutu-19 cells and 100% of EJ cells were killed even at a MOI of 20 with CD gene vectors.

Figure 5. Primary cell Cultures. Samples of tumor tissues and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy and cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation and the resulting cells cultured in RPMI1640 with 10% fetal serum. When the cells were at 80% confluency, the cells were infected in the flasks for 90 minutes with Ad-Lp-LacZ or Ad-CMV-LacZ virus vectors, after 48 hours incubation the positive cells were measured by X-Gal staining.

Figure 6. Ovarian Organ Cultures. Normal Ovarian tissue was obtained from operating room. The tissues were cut into small pieces and cultured in DMEM/Hamís F12 with 10% charcoal-stripped serum. 24-48 hours later, the tissues were infected with virus vectors for 90 minutes, PBS washed and then incubated 48 hours. Following infection, the tissues was frozen in O.C.T following which the frozen section were stained by X-Gal reaction. Left: control; Middle: Ad-CMV-LacZ; Left: Ad-Lp-LacZ.

Figure 7. Kaplan-Meir analysis of survival, Group 1: 22 mice received 1 to 2 million fresh marrow cells. Group 2:21 mice transplanted with 1 to 2 million marrow cells cultured in QBSF58 serum-free medium for 4 days, as was group 3, but not exposed to the CD adenoviral vector/5-FC. Group 3: 19 mice transplanted with 1 to 2 million marrow cells exposed to the CD adenoviral vector followed by 5-FC. Survival for each group was

estimated by the Kaplan-Meir model. Log-rank test was used to check for equality of survival functions. Sixty-two mice were studied. Follow-up was 83.9± 43.4 days (mean ± SD), with a range of 4 to 145 days. Survival functions: Results are given as the mean ± SE, with the 95% confidence interval in parentheses. Group 1: 22 mice were transplanted and 19 survived and 2 died at days 6 and 8 (2 different experiments). Survival: 90.5 ± 6.4 (67 to 97.5). Group 3: 19 mice were transplanted. Six died at days 4 (1 mouse), (4 different experiments total). Survival: 68.4 ± 10.7 (42.8 to 84.4). Log-rank test: P = .16. There was no difference in survival among these 3 groups.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. General Description

The present invention is based in part on the discovery that the plastin promoter may be used to effect tissue-specific transcriptional of a gene of interest in target tumor cells. The present inventors have discovered that a variety of neoplastic cells, including cell lines from ovarian cancer, breast cancer, bladder cancer as well as primary samples from surgical resections (ovarian cancer cells) can be sensitized to 5-Fluorocytosine (5FC) by vectors carrying the cytosine deaminase (CD) gene driven by the L-plastin promoter. Accordingly, the invention includes vectors and nucleic acid constructs comprising a plastin promoter and a gene of interest, particularly a gene encoding an enzyme for Gene Directed Enzyme-Prodrug Therapy (GDEPT).

In addition, when the L-plastin promoter is used to drive expression of cytosine deaminase, the amount of intracellular CD protein which is generated by the vectors is sufficient to kill 100% of the epithelial neoplastic cells, when only 5-50% of the cells are infected. Lastly, the present inventors have constructed conditionally replication competent vectors under the control of a plastin promoter for use in the *in vivo* treatment of cancer, such as the treatment of ovarian cancer by intraperitoneal delivery.

II. Specific Embodiments

L-plastin is an actin-binding protein selectively expressed at high levels in many human epithelial cancers. Vectors described herein are useful for the treatment of

metastatic diseases, particularly epithelial cell malignancies such as ovarian and breast cancer.

Plastin Promoters

The L-plastin gene belongs to a recently discovered gene family of actin-binding proteins (Leavitt *et al.*, 1994; Lin *et al.*, 1997; Park *et al.*, 1994). The only normal cell in which this protein is detectable is the leukocyte. This protein has been demonstrated to be present in greater than 90% of epithelial neoplastic cells, and is not found in normal epithelial cells.

As used herein, the term "plastin promoter" refers to any transcriptional regulatory element derived from the upstream or 5' region of a plastin gene, a plastin variant, or a gene that encodes a plastin-like protein. Also included are plastin promoter variants, for instance, sequence variants that exhibit tumor cell specific transcription. Preferably, regions upstream of the human L-plastin promoter are used to construct vectors of the invention. The sequence of the human L-plastin promoter region is readily available, for instance, as GenBank accession No. S54531. See also Lin *et al.* (1993b). Portions or fragments of the L-plastin promoter region may also be employed to conditionally express a gene of interest in neoplastic cells. For instance, a fragment spanning about the first 2265 nucleotides upstream of the L-plastin transcriptional start site may be used. In other instances, the progesterone- and/or estrogen-responsive elements may be used. Preferably, fragments of the upstream regions employed to construct vectors of the invention retain the inability to effect expression of the operably linked gene in normal non-neoplastic cells. See Lin *et al.* (1993a and 1993b) for the structure of the L-plastin promoter as well as useful fragments.

According to the invention, a coding sequence is "operably linked" to a plastin promoter when the promoter is capable of directing transcription of that coding sequence.

Viral Vectors

Any viral delivery vector or backbone may be used to target a gene of interest to a neoplastic cell, particularly a gene whose induction via a plastin promoter modifies the metabolism of a cell. For instance, many gene therapy vectors are available in non-

replicable, conditionally replicable and fully replicable forms as are vectors capable of specifically infecting tumor cells. See Alemany *et al.* (1999), Crystal (1999) and Zhang *et al.* (1999).

Retroviral vectors engineered to express a protein of interest from a plastin promoter can be used (see Miller *et. al.* (1993)). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. More detail about retroviral vectors can be found in Boesen *et al.* (1994)) which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et. al.* (1994); Kiem *et al.* (1994); Salmons and Gunzberg (1993); and Grossman and Wilson (1993).

Preferred delivery vectors include, but are not limited to, the many forms of recombinant adenoviruses currently available. Adenoviruses are especially attractive vehicles for delivering genes to epithelial cells. Adenoviruses naturally infect respiratory epithelia as well as other epithelia cells where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson (1993) present a review of adenovirus-based gene therapy. Bout *et al.* (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.* (1991); Rosenfeld *et al.* (1992); and Mastrangeli *et al.* (1993).

Conditionally replicative adenoviral vectors which replicate in neoplastic cells but not in normal cells are particularly useful for the delivery of proteins of interest under the control of a plastin promoter. Such conditionally replicative vectors may be produced by expressing the adenoviral E1A gene. In a preferred embodiment, the E1A gene is under the transcriptional control of a tissue-specific and/or disease-specific promoter. In a most preferred embodiment, the E1A gene is expressed under the control of a plastin promoter as described in the Examples.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy

(Walsh *et al.* (1993)).

Prodrug Activation Systems and Gene Therapy Approaches

The vectors of the invention can be used to express any gene of interest, including therapeutic genes or reporter genes. For instance, a therapeutic gene includes genes which exert their effect at the level of RNA or protein. The protein encoded by the therapeutic gene may exert its therapeutic effect by cell killing. For instance, expression of the gene in itself may lead to cell killing, as with expression of the diphtheria toxin A gene, or the expression of the gene may render cells selectively sensitive to the killing action of certain drugs. As the L-plastin promoter is transcriptionally active specifically in neoplastic cells, vectors of the invention comprising a L-plastin promoter operably linked to a gene or genes which kill or in some way inhibit the growth of neoplastic, cancer or tumor cells are preferred embodiments.

The therapeutic gene can also exert its effect at the level of RNA, for instance, by encoding an antisense message or ribozyme, a protein which affects splicing or 3' processing (*e.g.*, polyadenylation), or by encoding a protein which acts by affecting the level of expression of another gene within the cell (*i.e.*, where gene expression is broadly considered to include all steps from initiation of transcription through production of a processed protein), perhaps, among other things, by mediating an altered rate of mRNA accumulation, an alteration of mRNA transport, and/or a change in post-transcriptional regulation.

Accordingly, the use of the term "therapeutic gene" is intended to encompass these and any other embodiments commonly referred to as gene therapy and is known to those of skill in the art. Similarly, the recombinant adenovirus can be used for gene therapy or to study the effects of expression of the gene in a given cell or tissue *in vitro* or *in vivo*.

In one embodiment, the enzyme component of Gene-Directed Enzyme-Prodrug Therapy (GDEPT) may be expressed by the vectors of the invention. GDEPT is an approach where the tumor-specific activation of pharmacologically inert compounds (prodrugs), by either endogenous or specifically introduced exogenous enzymes, brings tissue selectivity to cancer chemotherapy.

Numerous prodrug systems have been developed for GDEPT and the vectors of the invention may be used to specifically deliver and express the enzymes in each of these systems. Table A summarizes these prodrug systems (Patterson *et al.* (1999)).

Table A

Prodrug	Enzyme	Reference
6-Methoxypurine arabinonucleoside	Varicella Zoster thymidine kinase	Huber *et al.* (1991)
Purine nucleosides, including acyclovir, ganciclovir and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil	Herpes simplex thymidine kinase	Moolten *et al.* (1986)
6-Methylpurine-2'-deoxyribonucleotide	*E. coli* purine nucleotide phosphorylase	Sorscher *et al.* (1994)
5-Fluorocytosine	*E. coli* cytosine deaminase	Mullen *et al.* (1992) and Huber *et al.* (1993)
Dinitrobenzamide mustard derivatives	*E. coli* nitroreductase	Bridgewater *et al.* (1995), Bailey *et al.* (1996) and Friedlos *et al.* (1997)
5'-Galactosylates of 1-β-D-arabinofuranosylcytosine and 1-β-D-deoxyribofuranosylcytosine	*E. coli* β-galactosidase	Douglas *et al.* (1991)
Cyclophosphamide and ifosfamide	Rat or human cytochrome P450 2B and 3A	Chen *et al.* (1996)
Dinitrobenzamide mustard derivatives	Human NADPH; cytochrome P450 reductase	Walton *et al.* (1989)
1-β-D-arabinofuranosylcytosine	Human deoxycytidine kinase	Manome *et al.* (1996)
5'-deoxy-5-fluorouridine	Human thymidine phosphorylase	Patterson *et al.* (1995)
irinotecan	carboxylesterase	Crystal (1999)

As disclosed in the Examples, the invention includes, but is not limited to vectors and methods using cytosine deaminase as the enzyme component of GDEPT and 5-

Fluorocytosine as the prodrug component. As used herein, however, a "gene whose induction modifies the metabolism of a cell" includes any gene capable of in some way beneficially modifying or modulating the metabolism of the target cell upon infection by the vectors of the invention.

Vectors of the invention for GDEPT therapy may also include other optional genes. For instance, at least one copy of a gene encoding orotate phosphoribosyltransferase (OPRTase) may be included in vectors of the invention to increase the rate of phosphorylation of a fluoropyrimidine and to increase the rate of incorporation into nucleic acids as is known in the art.

Pharmaceutical Compositions

The vectors of the present invention can be employed to contact cells either *in vitro* or *in vivo*. According to the invention "contacting" comprises any means by which a vector is introduced intracellularly; the method is not dependent on any particular means of introduction and is not to be so construed. Means of introduction are well known to those skilled in the art, and also are exemplified herein.

Accordingly, introduction can be effected, for instance, either *in vitro* (*e.g.*, in an *ex vivo* type method of gene therapy or in tissue culture studies) or by electroporation, transformation, transduction, conjugation or triparental mating, (co-)transfection, (co-)infection, membrane fusion with cationic lipids, high velocity bombardment with DNA-coated microprojectiles, incubation with calcium phosphate-DNA precipitate, direct microinjection into single cells, and the like. Similarly, the vectors can be introduced by means of cationic lipids, *e.g.*, liposomes. Such liposomes are commercially available (*e.g.*, Lipofectin™ , Lipofectamine™ , and the like, supplied by Life Technologies, Gibco BRL, Gaithersburg, Md.). Moreover, liposomes having increased transfer capacity and/or reduced toxicity *in vivo* (see, *e.g.*, PCT patent application WO 95/21259) can be employed in the present invention. Other methods also are available and are known to those skilled in the art.

According to the invention, a "host" (and thus a "cell" from a host) encompasses any host into which a vector or recombinant virus of the invention can be introduced, and

thus encompasses an animal, including, but not limited to, a mammal. Optimally a host is a human.

One skilled in the art will appreciate that suitable methods of administering a vector (particularly an adenoviral vector) of the present invention to an animal for purposes of gene therapy (see, for example, Rosenfeld *et al.* (1991a); Jaffe *et al.* (1991); Rosenfeld *et al.* (1991b); and Berkner. (1988)), chemotherapy, and vaccination are available, and, although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Pharmaceutically acceptable excipients also are well-known to those who are skilled in the art and are readily available. The choice of excipient will be determined in part by the particular method used to administer the recombinant vector. Accordingly, there is a wide variety of suitable formulations for use in the context of the present invention. The following methods and excipients are merely exemplary and are in no way limiting.

As discussed in the Examples, recombinant viral vectors of the invention may be formulated to achieve a desirable infection rate depending on the tissue, tumor mass, location of tumor, enzyme expressed, etc. For instance, *in vivo* delivery vectors comprising a plastin promoter operably linked to a gene whose induction modifies the metabolism of a cell may be formulated to achieve an infection rate in neoplastic or tumor cells of about 1 or 2%, about 5%, about 10%, about 15%, about 30%, about 50% or about 70% or more, up to an infection rate of about 100%. In vectors to be used in GDEPT protocols where cytosine deaminase is the enzyme encoded by a vector of the invention and wherein the prodrug is 5-FC, formulations may be prepared to achieve killing of 100% of all of the epithelial neoplastic cell lines, when only about 5-50% of the cells are infected.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide,

croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible excipients.

A vector or transfer vector of the present invention, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They may also be formulated as pharmaceuticals for non-pressured preparations such as in a nebulizer or an atomizer.

Formulations suitable for parenteral administration (including implants) include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

Additionally, a vector of the present invention can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

The dose administered to an animal, particularly a human, in the context of the present invention will vary with the gene of interest, the composition employed, the method of administration, and the particular site and organism being treated. However, the dose should be sufficient to effect a therapeutic response.

Vectors or nucleic acid constructs of the invention to be used in GDEPT protocols may also be packaged with the relevant prodrug and with instructions. The packaged

formulations may include a pharmaceutical composition of the invention in a container with printed instructions for administration of the composition to a subject or patient. In another format, the packaged formulation may contain a pharmaceutical composition with general written material indicating or suggesting the use of the composition and any other compounds or formulations contained therein for treating a patient diagnosed with cancer. As discussed in the Examples, the vectors, compositions and methods of the invention are particularly useful for the treatment of epithelial cell derived malignancies, including but not limited to, ovarian cancers, breast cancers, bladder cancers and the like.

Methods of Purging Transplantation Samples

Currently, cancer patients with gross contamination of the bone marrow with tumor cells may be excluded from autologous stem cell transplantation. For instance, PCR studies that identify markers for metastatic circulating cells indicate that advanced disease breast cancer patients have cancer cells contaminating their bone marrow (Garcia-Sanchez, 1998). Accordingly, there has been an increased interest in procedures that can selectively remove cancer cells from autologous hematopoietic cells before transplantation.

The vectors and methods of the invention are useful tools to eradicate the presence of malignant cells from transplant samples, including autologous hematopoietic cells. For instance, vectors of the invention can be used to infect malignant cells contaminating hematopoietic reconstituting cells before introduction into the patient. The skilled artisan can modify any of the available malignant cell purging methods to utilize the vectors, viruses, compositions and methods of the invention. Such methods include, but are not limited to, the methods discussed by Gee *et al.* (1995), Hammert *et al.* (1997), Spitzer *et al.* (1996) and Brugger *et al.* (1997).

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the vector and compositions of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

The following materials and methods were employed in the Examples:

Cells and Cell Culture:

Human bladder carcinoma cell lines (J82, EJ) were obtained from The Department of Pathology of the University of Southern California School of Medicine in Los Angeles, CA. The CCD minimal deviation human fibroblast cell and the 293 transformed human kidney cell line were obtained from the American Type Culture Collection (ATCC). The Hey cystadenocarcinoma papillary ovarian carcinoma cell line was obtained from the Department of Therapeutic Radiology of the Yale University School of Medicine. All of these cells were propagated in DMEM (GIBCO-BRL) supplemented with 10% heat-inactivated new born calf serum (Hyclone Laboratories Inc., Logan. UT).

The Nutu-19 rat ovary adenocarcinoma cell line, which was derived from a poorly differentiated adenocarcinoma, and the Ovcar-5 human epithelial ovary carcinoma cell line, were both obtained from the Department of Medical Oncology at the Fox Chase Cancer Center, Philadelphia, PA. These cells were grown in RMPI-1640 (GIBCO-BRL) supplemented with 10% heat-inactivated new born calf serum.

The human ovary adenocarcinoma cell line (Skov3) was purchased from ATCC and was propagated in McCoy 5A Medium supplemented with 10% heat-inactivated new born calf serum. All cell cultures were maintained in a 5% CO_2 humidified tissue culture incubator at 37°C.

Chemicals and Reagents:

5-FC, 5FU, fluorescein di-β-D-galactopyranoside (FDG), X-gal (4-bromo-5-chloro-3-indoyl-β-galactopyrosanide) were all purchased from Sigma Chemical Co. 6-3(H)5-Fluorocytosine(4.1Ci/μmol) and 6-3(H)5-Fluorouracil were purchased from Noravek Biochemicals Inc. of Brea, CA. Monoclonal antibodies to αVβ3 (LM609) and αVβ5(P1F6) integrins were purchased from Chemicon International. A monoclonal antibody to the Coxsackie virus receptor (CAR), which binds the adenoviral fibrillar protein, was obtained from the Dana-Farber Cancer Institute, Harvard Medical School, Boston, Mass.

Construction of Replication Incompetent Recombinant Adenoviral Vectors:

A replication-incompetent recombinant adenoviral vector was obtained from the Cornell Medical School, New York, NY. This vector (Ad-CMV-CD) contained the cytosine deaminase gene controlled by a cytomegalovirus (CMV) promoter (Hirschowitz (1995)). In this vector, a portion of the E1a and E1b gene region of human adenovirus serotype 5 had been replaced by the bacterial cytosine deaminase gene which was placed under the transcriptional control of the CMV promoter as described previously. A similar adenoviral vector (Ad-CMV-LacZ) was engineered in our laboratory in which a β-galactosidase transcriptional unit was inserted into the E1a and E1b regions (Garcia-Sanchez *et al*., 1998).

The 5Kb L-plastin promoter was truncated to a 2.4Kb fragment, which extends from nucleotide -2265 of the 5' region of the L-plastin promoter to +18bp from the transcription initiation site. The original human L-plastin promoter was derived from the PHLPr-β-gal-Neo plasmid (Lin *et al*., 1993, 1993b). This truncated L-plastin promoter was used to replace the CMV promoter in the Ad-CMV-CD adenoviral vector, which then generates the Ad-Lp-CD and Ad-Lp-LacZ viral vectors. The recombinant adenoviral vectors were then purified by cesium chloride (CsCl) gradient density centrifugation. The final viral band was diluted 1:1 with sterile glycerol and stored at -70°C . The number of infectious adenoviral particles, expressed as plaque-forming units (pfu), present in the viral stocks was determined by limiting dilution assay of plaque formation in 293 cells exposed to various dilutions of the vector (Graham *et al*., 1991; Neer *et al*., 1996).

Analysis of Cellular Receptors on Tumor Cells which Participate in Vector Uptake:

Mouse monoclonal antibodies to the αVβ3 [LM609] integrin, αVβ5 [P1F6] integrin, and to the CAR receptors were used to detect the density of the human αVβ3, αVβ5 and CAR receptors on the cells. J82, EJ, Ovcar-5, Nutu-19, Hey, Skov3 and CCD cells were stained with each monoclonal antibody using standard procedures. The FACS Star Flow Cytometer (Becton Dickinson) in Yale Cancer Center was used to determine the percentage of cell positive for each receptor.

β-galactosidase Activity Assay:

X-gal staining:

Cells were washed in phosphate buffered saline (PBS), trypsinized, and the viable cell number counted by Trypan Blue exclusion, using a light microscope. $2x10^5$ cells for each cell line were infected with varying ratios of plaque forming units (pfu) per cell (MOI) in DMEM supplemented with 2% serum for 90 minutes. Following this, the cells were plated in 6 well plates in complete medium in duplicate cultures. Following 48 hours of incubation at 37°C in a 5% CO2 humidified tissue culture incubator, the cells were fixed with ice-cold 2% paraformaldehyde/0.2% glutaraldehyde for 10 minutes. The level of β-gal expression was then assessed by staining the cultures with X-Gal and potassium-ferrcyanide/ferrocyanide solution essentially as described previously (Couffinhal *et al.*, 1997; Lawrence *et al.*, 1998). The average number of β-gal expressing (blue) cells per well was determined by counting 5 separate microscopic high-power fields and then the average of the 5 fields determined.

Fluorescein FDG Flow Cytometry Analysis of LacZ Report Gene Expression in Cells Exposed to the Ad-CMV-LacZ and Ad-Lp-LacZ Vectors:

$3x10^5$ cells were exposed for 90 minutes to the Ad-CMV-LacZ or Ad-Lp-LacZ vectors at different MOI in DMEM medium supplemented with 2% serum and then incubated in complete medium for 48 hours in 6 well plates in triplicate. Following this, the cells were subjected to FACS-gal analysis as described by Roederer *et al.* (1991). Briefly, after harvesting by centrifugation, the cells were resuspended in 50μl of staining medium (RPMI 1640 supplemented with 4% FBS and 10 mM Hepes, pH 7.4) containing chloroquine, and incubated for 20 minutes at 37°C. The cells were then exposed to 50μl of 2mM fluorescein di-β-d-galactopyranoside (FDG) for 60 seconds, following which 1ml of ice-cold quench solution (staining solution with propidium iodide) was added and the cells which were then further incubated on ice for 1 hour. After this, phenylethyl β-D-thiogalactopyranoside (PETG) was added to stop the reaction. Then, the cells were subjected to FACS analysis.

The Effect of 5-FU Released from Cytosine Deaminase Vector Infected Cells on Uninfected Cells:

To quantify the effect of 5-FU released from infected cells on uninfected cells, different cell lines were infected at varying MOI (20MOI, 80MOI, 160MOI) using the Ad-CMV-CD or Ad-Lp-CD vectors. The infected cells and non-infected cells were mixed in varying ratios to generate 0, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 100% of infected cells. Cells then were seeded in triplicate in six well tissue culture plates and incubated for 24 hours. This was followed by incubation with 500μmol/L 5FC for 6 days. Cells were then washed with PBS. The number of surviving cells was determined using Trypan Blue exclusion (Kianmanesh *et al.*, 1997).

Comparison of the 5-FU Sensitivity (IC50) of Ovarian Cancer and Bladder Cell Lines with CCD(Minimal Deviation Fibroblast Cell Lines):

Each cell line was seeded at 3.0×10^5 cells/ml in 25cm^2 flasks. There were 10ml of medium per flask. The flasks were placed at 37°C in a 5% CO_2 tissue culture incubator for 24 hours to allow the cells to attach to the flask surface before treatment with 5-FU. Sufficient numbers of cells were prepared for triplicate assay of experimental samples and a triplicate control. After 24 hours, cells were treated with the appropriate dose of 5FU, with the exception of the controls. All the flasks were placed in the incubator for 96 hours. The concentrations of 5FU used to test the IC50 were 100, 50, 10, 1, and 0.5µM. After 96 hours, the cells were removed with trypsin-EDTA and cell density calculated using the Coulter Counter ZM (Hialeah, Fl).

The Toxicity of Adenoviral Vectors:

2×10^5 cells were infected with the Ad-CMV-LacZ, Ad-Lp-LacZ, Ad-CMV-CD, or Ad-Lp-CD vectors at a MOI of 0, 5, 20, 40, 80 and 160 for 90 minutes, and then seeded in six well plates in duplicate. 24 hours later, 0.5mM 5FC was added to each well, and then incubated for 5 days. The cells were then trypsinized and the surviving cells were counted using Trypan Blue exclusion. A 100% value was assigned to the cells incubated at 0 MOI and the percentage of viable cells in the cultures to which vector had been added was calculated.

Vector Studies in Primary Cell Monolayer Culture:

Samples of primary tumor, metastatic tumor, and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy and cut into small pieces. These pieces were then digested with collagenase to disaggregate the tissue and the resulting cells cultured in RPMI 1640 supplemented with 10% NCS. Samples of ascites were divided into the T25 flasks directly and washed to remove debris after cell attachment. All experiments were performed at 80% confluency. To measure the percentage of cell expressing β-galactosidase in the sample, the Ad-CMV-LacZ or Ad-Lp-LacZ vectors were added to the flasks for 90 minutes in DMEM supplemented with 2% serum. After 48 hours of incubation in complete medium, the cells were studied for infectivity by the vectors by X-gal staining or FACS. To test the sensitivity of the patient samples to infection with the Ad-CMV-CD and Ad-Lp-CD vectors, the cells were grown in T25 flasks until 80% confluency. The cells were then washed in PBS and then exposed to vector directly in the flasks containing DMEM supplemented with 2% serum for 90 minutes. The cells were then incubated for 5 days at 500μmol/L 5FC concentration and then the cell viability was determined by light microscopic examination.

Vector Studies on Primary Cell Organ Culture of Ovarian Cancer Cells and Normal Ovarian Tissue:

Normal human ovarian and ovarian tumor tissues were obtained from the operating room under protocols approved by IRB of the Yale School of Medicine. Immediately following excision from the patients, the tissues were washed with minimal essential medium (DMEM/Ham's F12). Each specimen was then cut into pieces of approximately 1-2 mm^3, and immersed in 4ml of DMEM/Ham's F12 medium, which was supplemented with 10% charcoal-stripped serum (Sape *et al.*, 1998). Cultures were incubated at 37°C in 6 well plates on a shaking platform for 24-48 hours, after which, the tissues were exposed to the Ad-CMV-LacZ, Ad-LP-lacZ, Ad-CMV-CD or Ad-LP-CD viral vectors for 90 minutes in serum free medium. The tissues were then washed with PBS. The Ad-CMV-CD and Ad-LP-CD viral vectors containing wells were then incubated with 0.5 mM 5-FC, at 48-72 hours of incubation. The cultured tissues were then fixed in O.C.T. X-Gal staining was used to measure the Ad-CMV-LacZ and Ad-LP-LacZ expression on slides.

The AD-LP-CD and Ad-CMV-CD infected tissues were also fixed in O.C.T. and stained with hematoxylin/eosin. The structure and morphology were observed on slides.

Vector Studies in SCID Mice:

Ovcar-5 tumor cells were infected at 100 MOI with either the Ad-LP-LacZ or Ad-Lp-CD viral vectors for 60 minutes, then washed with PBS, and then suspended in PBS ($4x10^7$ cells/1ml PBS). Ten 6-8 week old female SCID mice (25-28 grams in weight), which were purchased from Cox, Inc., Cambridge, MA, were injected intraperitoneally with one million Ovcar-5 ovarian carcinoma cells previously infected at 100 MOI with the Ad-Lp-LacZ vector (Molpus *et al.*, 1996). An additional ten 6-8 week old 26-28 gram mice were injected intraperitoneally with Ad-LP-CD infected cells. From the second day, all of the 20 mice were injected once a day with 5-FC in 500 mg/kg intraperitoneally for 10 days. After 3 weeks of tumor cell injection, the 10 Ad-LP-LacZ injected mice and the 7 Ad-LP-CD injected mice were sacrificed and autopsied. At the 50th day, another 3 Ad-LP-CD injected mice were sacrificed and autopsied.

Another 10 female Swiss nude mice of 6-8 weeks old (26-28 grams) were injected with one million Skov3 ovarian adenocarcinoma cells. 5 mice were injected with Skov 3 cells infected with Ad-Lp-CD at 80 MOI. 5 mice were injected Skov 3 cells infected at 80 MOI with the Ad-LP-LacZ vectors ($4x10^7$ cell/ml PBS per mouse). Then, 500mg/kg 5FC were injected intraperitoneally daily for 10 days into the 10 mice. 3 weeks later, the mice were sacrificed and autopsied.

<u>Example 1</u>

Correlation of Infectivity by Adenoviral Vectors and Levels of CAR and Integrin Receptor Expression in Cancer and Minimal Deviation Cell Lines

The integrin $\alpha V\beta 5$ receptor, and the coxsackie adenovirus receptor (CAR) have been reported to play an important role in adenovirus infection (Bergelson *et al.*, 1997,1998; Roelvink *et al.*, 1998, Wickham *et al.*, 1993,1994). Differences in the integrin receptor expression levels and the functional state of these receptors may explain differences in the sensitivity to infection by the adenoviral vector observed in established tumor cell lines. Therefore, the level of expression of the integrin and CAR receptors was

evaluated in the Nutu-19, Ovcar-5, Skov3, Hey, EJ, J82, and C CDD cell lines (Table1). The alpha V beta 5 and CAR receptor levels appeared in our data to correlate with the ability of cells to take up the Ad-CMV-LacZ vector and to express the Lac Z reporter gene. For instance, very low levels of LacZ expression were observed in the Hey cell line in which the CAR was undetectable. The low level of CAR in Nutu-19 is an artifact due to the non reactivity of the antibody to the human CAR with the rat CAR. The rest of the cell lines which were infectable by the Ad-CMV-LacZ, as judged by the high level LacZ expression, had substantial levels of CAR detectable. This suggested that the CAR receptor was important to vector infection in cell lines.

It was also discovered that low levels of the $\alpha V\beta 3$ and $\alpha V\beta 5$ receptors in the cell lines with lower level of LacZ expression were seen when we restrict our analysis to cell lines with substantial level of CAR (Ovcar-5, Skov-3, EJ, J82). The low level of the $\alpha V\beta 3$ in Nutu-19 cell line may again reflect the divergence of the rat receptor from which of the human receptor, making the detection of this receptor in the rat cell with an anti-human antibody difficult. Thus, the most important receptors for Ad-CMV-LacZ infectivity and transgene expression appeared to be the CAR and alpha V integrin receptors.

Example 2

Comparison of Lac Z Gene Expression Levels in Cell Lines Infected with the Ad-CMV-LacZ and Ad-Lp-LacZ Vectors:

In order to determine if the L-plastin promoter was selectively more active in epithelial neoplastic (ovarian and bladder cancer) cell lines than in minimal deviation fibroblast cell line (CCD), we tested for the percentage of cells in which the presence of LacZ was detectable in the Nutu-19, Ovcar-5, EJ, J82, Skov3, Hey and CCD cell lines following exposure to the replication incompetent adenoviral vectors which carry the LacZ transcription unit driven either by the L-plastin promoter (Ad-Lp-LacZ) or the CMV promoter (Ad-Lp-LacZ). We then calculated a ratio of the percentage of cells positive for LacZ in the CMV to the LP transcription unit vectors, to normalize the data for differences in each cell line. As shown in Table 2, the ratio of CMV to LP expression 5 days following exposure to each vector at 80 MOI was high (10/1) with the CCD as compared

to the epithelial neoplastic cell lines (4/1, 1/1, 1.4/1, and 1/1). These data suggest that the L-plastin promoter is more active in epithelial neoplastic cell lines than in minimal deviation fibroblast cell lines. This data is also presented in Figure 1, in which the percentage of cells positive for LacZ in the CCD cell line is compared to the EJ bladder cancer cell line.

Table 2. The Positive Percentage of LacZ Gene Expression

	Ad-CMV-LacZ				Ad-Lp-LacZ			
	Day3		Day5		Day3		Day5	
	20MOI	80MOI	20MOI	80MOI	20MOI	80MOI	20MOI	80MOI
Ovcar-5	65	80	10	35	10	35	1	8
Skov3	85	95	15	40	25	60	8	35
Hey	10	35	5	10	0	2	0	0
EJ	95	100	45	85	55	95	45	60
J82	90	100	15	35	20	55	7	35
CCD	70	95	12	55	3	8	2	5

Light Microscopic Study of the LacZ gene expression levels in different cell lines and time. $1.4x10^5$ cells were infected at 20 and 80 MOI with Ad-LP-LacZ or Ad-CMV-LacZ virus vector for 90 minutes. Then the cells were incubated in duplicate 6 well plates and after 48 and 96 hours the X-Gal staining was performed. The positive cells were counted in the high powered field of the light microscope (percentage).

Example 3

Time Course of Expression of Adenoviral Transgenes after Infection:

Cells were washed in phosphate saline buffer , and the cell number was determined by Trypan Blue exclusion. The Ad-CMV-LacZ and Ad-LP-LacZ vectors were used at different MOI to infect $6x10^5$ cells for 90 minutes. The cells were then seeded in 6 well plates in duplicate and then stained by the X-Gal reaction at 2, 3, 4, 5, 6, and 7 days following the infection to measure the positive cells. The expression of the transgene following exposure to the Ad-CMV-LacZ vector quickly reached a maximum and persisted at that level for 3-5 days (80MOI) before it started to decrease. When a lower

MOI (5MOI) was used, the maximum expression level persisted in a shorter time. Cells infected with the Ad-Lp-LacZ vector reached a maximum level of expression much later but ultimately reached a maximum expression at day 3 (80MOI). In the EJ cell line, the L-plastin driven LacZ gene cell line reached an expression level that was as high as seen when the vector carrying the CMV promoter was used (see Figure 2).

Example 4

Studies in Cell Lines of the Effect of 5-FU Released from Infected Cells on Non-Infected Cells:

In order to monitor the effect of 5FU released from infected cells on the survival of non-infected cells, mixtures of infected and non-infected cells were generated and then exposed to 5FC. When as few as 5% of the population of cells infected with Ad-CMV-CD (20MOI) vectors were mixed with 95% of uninfected cells, the majority of the cell lines exhibited complete eradication when cells were exposed for 6 days to 5FC at a 500μmol concentration (see Figure 3A). This suggests that only a few of these cells need to be infected with the Ad-CMV-CD adenoviral vector to generate sufficient levels of 5FU which are required to kill the vast majority of uninfected tumor cells. Figure 3B, the EJ cells can reach almost the same level of cell kill with the Ad-Lp-CD vector as with the Ad-CMV-CD vector. This suggests in this cell line, that the LP promoter generates a peak level of CD expression that is equivalent to that the EJ cells can reach almost the same level of cell kill with the Ad-Lp-CD vector as with the Ad-CMV-CD vector. This suggests in this cell line, that the LP promoter generates a peak level of CD gene expression that is equivalent to that reached with the CMV promoter. In some cell lines which were less infectable with the adenoviral vectors (see Table 1), an increase in the MOI was required to achieve the same level of cell kill with the Ad-Lp-CD as compared with the Ad-CMV-CD (*e.g.* Ovcar-5 in Figure 3A). However, no matter how high the MOI was taken, 100% cell kill was never reached with the Ad-Lp-CMV vector with the CCD cell line, whereas 100% cell kill was achievable with the Ad-Lp-CD vector in all of the epithelial neoplastic cell lines tested (see Figure 3A).

Table 1. The Relationship Between CAR, αVβ3, αVβ5 Expression Level and Infectivity by the Ad-CMV- LacZ Vector as Measured by LacZ Expression

	αVβ3	αVβ5	CAR	Ad-CMV-LacZ
Ovarian Cancer Cell Lines:				
Nutu-19	17.37	93.79	10.2*	100
Ovcar-5	47.71	57.15	88.07	65
Skov3	63.75	91.2	87.25	85
Hey	81.3	96.3	0.0	10
Bladder Cancer Cell Lines:				
EJ	82.73	81.53	94.7	95
J82	55.8	77.9	80.3	88
Minimal Deviation Cell Line:				
CCD	62.8	92.8	29	70

αVβ3, αVβ5 and CAR (coxsackievirus-adenovirus receptor) monoclonal antibodies and the FITC conjugated Anti-Mouse antibody were used to stain the cells. Then, FACS analysis was used to detect the percentage of the positive cells. *Low value probably due to nonreactivity of the mouse antihuman CAR monoclonal antibody used.

Example 5

Study of the Toxicity of the Vector Backbone:

In order to test how much of the toxicity seen in Figure 3 was due to the vector backbone and how much was due to the CD transcription unit, the cell lines were first infected with the AD-CMV-CD, Ad-CMV-LacZ, Ad-Lp-CD or Ad-Lp-LacZ vectors at different MOI. Following this, the cell lines were incubated in medium supplemented with 500μM 5-FC for 5 days. As shown in Figure 4, no toxicity was seen at an 80 MOI with the Ad-CMV-LacZ or Ad-Lp-LacZ vectors. In contrast, 100% of Nutu-19 cells and 100% of EJ cells were killed even at a MOI of 20 with the Ad-Lp-CD vectors (see Figures 3 and 4). 50% of Ovcar-5 were killed at a MOI of 20. Thus, the toxicity seen in these experiments was not due to the viral backbone, but due to the effect of the CD transcription units on the conversion of 5FC to 5FU. In contrast, 50% of the CCD cells survived, even at a MOI of 160, when the Ad-LP-CD vector was used (see Figure 4). Thus, greater cell kill which was specific to the Ad-Lp-CD/5FC system was seen in the ovarian carcinoma cell lines as compared to the CCD fibroblast cell line.

Example 6

5FU Sensitivity of Each Cell Line Expressed as IC50:

Since the sensitivity to the Ad-Lp-CD vector/5 FC system might be due to the intrinsic sensitivity of each cell line to 5FU and not due to the level of expression of Lp regulated genes, we decided to test the intrinsic sensitivity of each cell line to 5FU. 3×10^5 cells were seeded in T25 flasks in triplicate, and incubated for 96 hours at different 5FU concentrations. The number of cells was remaining was calculated using a Coulter Counter. The IC50 to 5FU for J82 is 55μM, EJ is 30μM, Nutu-19 is 5μM, Ovcar-5 is 3μM, Skov3 is 22μM , Hey is 85μM and CCD is 15μM. The resistance of the CCD fibroblast cell line to the effect of the Ad-Lp-CD vector with 5FC treated is not due to a high level of resistance to 5FU, since this cell line has one of the lowest IC50 to 5FU. Thus, the resistance of this cell line to 5FC sensitization with the Ad-Lp-CD vector is due to the low level of CD expression with the Lp promoter in this cell line.

Example 7

LacZ Expression in Monolayer Cultures of Samples Obtained at Surgery from Normal Peritoneum and Metastatic Implants of Ovarian Cancer Following Exposure to Ad-Lp-LacZ and Ad-CMV-LacZ Vectors:

Samples of metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients undergoing diagnostic or therapeutic laparotomy. This tumor was cut into small pieces. These pieces were then digested with collagenase to dissagregate the tissue. The resulting cells were then cultured in RPMI-1640 supplemented with 10% NCS. Following cell culture, cells were exposed at a MOI of 20 to the Ad-CMV-LacZ vectors in the flasks for 90 minutes. After 48 hours incubation, the infected cells were measured by X-gal staining or FACS. All experiments were performed at 80% confluency. As shown in Figure 5, staining of these cultures *in situ* showed blue color in all but the culture of normal peritoneum which had been exposed to the Ad-Lp-LacZ vector.

The percentage of X-gal positive cells in different patient samples following exposure to the Ad-CMV-LacZ vector, as determined by FACS, was calculated to be: ascites: 50-80%; primary tumor: 50-90%; metastatic tumor: 45-85%; and normal peritoneum: 60%. When cells exposed to the Ad-Lp-LacZ at a MOI of 80, the X-Gal staining in ascites was 10-35%; primary tumor 15-60%; metastatic tumor 15-45%; and normal peritoneum 1%. As shown in Table 3, the ratio of X-gal staining with CMV/LP vectors was highest with the normal peritoneum (60/1), whereas this ratio was in the range of 1/1 to 3/1 in the case of ovarian cancer and peritoneum or mesothelia analysis. This indicates that the normal peritoneum is less able to support the expression of transgenes driven by the LP promoter than in ovarian cancer cells.

Table 3. LacZ Transgene Expression Efficiency in Primary Culture of Ovarian Cancers

		Ascites	Primary Tumor	Metastatic Tumor	Normal Peritoneum
βgal	X-Gal	50-80%	50-90%	45-85%	60-80%
	FACS	95%	94%	94%	
βgal	X-Gal	10-35%	15-60%	15-45%	1-4%
	FACS	39%	83%	38%	

Samples of primary tumor, metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patient undergoing diagnostic or therapeutic laparotomy and cut into small pieces. These pieces were then digested with collagenase to produce tissue disaggregation and the resulting cells cultured in RPMI with 10% NCS. All experiments were performed at 80% confluency. Samples of ascites were divided into the T25 flasks directly and washed to remove debris after cell attachment. Cells were infected in the flasks for 90 minutes, after 48 hours incubation the positive cells were measured by X-gal staining or FACS.

Example 8

Cytotoxicity of Ad-CMV-CD and Ad-Lp-CD Vectors in Monolayer Cell Culture of Normal Peritoneum and Ovarian Cancer from Surgical Specimens:

Samples of primary tumor, metastatic tumor and normal peritoneum were collected from 16 ovarian cancer patients, and samples were prepared with the same methods as described previously. As shown in Table 4, when the cells were infected with the CD vectors and incubated for 5 days in the presence of 500μM 5FC in T25 flasks, 98% ascitic cells with Ad-CMV-CD and 85% were killed with Ad-Lp-CD vector. In the primary tumor cells, 90% of the cells were killed with Ad-CMV-CD vector and 75% were killed with Ad-Lp-CD vector. In metastatic cells, 85% were killed by the Ad-CMV-CD vector and 70% were killed by the Ad-Lp-CD vector. When the patient sample cells were grown

in T25 flasks and infected with the CD vectors at an 80 MOI and incubated in 500µmol 5FC, the 5FC to 5FU conversion was evaluated by HPLC. The 5FU conversion concentration reached about 400µmol with the Ad-CMV-CD vector and 20µmol with the Ad-Lp-CD vector. This data suggests that the LP vectors will be useful since the IC50s of most primary ovarian cancer cells is in the 5 µmol range.

Table 4. Cytotoxicity in Monolayer Culture of Normal Peritoneum and Ovarian Cancer Cells Following Expression to Ad-Lp-CD and Ad-CMV-CD Vectors and 5FC

	Ad-CMV-CD	Ad-Lp-CD
Ascites	98%	85%
Metastatic Tumor	85%	70%
5-FU concentration	400µmol	20µmol

In Ad-CMV-CD and Ad-Lp-CD infected samples, 500µMol 5-FC were added and incubated for 5 days, then the cells killing percentage were estimated by compared with the uninfected control flasks. The 5-FC to 5-FU conversion was done in metastatic tumor cells with 500µmol of 5-FC incubated for 5 days and the HPLC was used to test the 5-FU concentration.

<u>Example 9</u>

Studies of CD and LacZ Vectors in Organ Cultures of Normal Ovary:

The samples of normal ovary and ovarian carcinoma and normal peritoneum were cut into small pieces and were then cultured for 24-48 hours, infected with either the Ad-CMV-LacZ or the Ad-LP-LacZ vectors for 48 hours, and then the cells were processed with the X-gal staining reaction. As shown in (Figure 6), there is much stronger blue staining in the outer edges of the cell mass in the organ cultures of normal ovarian tissue with the Ad-CMV-LacZ vector than with the Ad-Lp-LacZ vector.

<u>Example 10</u>

Killing of Ovarian Cancer Tumor Cell Lines by 5FC/CD Vector System in Nude Mice:

In order to test the in vivo efficacy of the Ad-Lp-CD replication incompetent vector system, we infected either the Skov3 at 80 MOI or the Ovcar-5 cell lines *ex vivo* at 100 MOI by incubating the cells for 60 minutes. Then we injected 40 million of these infected cells into either 10 SCID mice with Ovcar-5 or 5 SCID mice with Skov3 ovarian carcinoma cell lines. One day after the injection of the tumor cells, we initiated daily intraperitoneal injections of each of the animals with 500mg/kg 5FC to generate peak intraperitoneal 5FC concentrations. We carried on the daily intraperitoneal injections for 10 days after the tumor injection. We then sacrificed 7 of the Ovcar-5 injected mice and all 5 of the Skov3 injected mice and examined the peritoneal cavity for tumors at day 21.

The remaining 3 Ovcar-5 mice were not sacrificed at 21 days were sacrificed at 50th days after tumor injection. All of these animals were free of detectable tumor nodules, either at the gross morphological level or at the histopathological level. The peritoneal surface was totally normal in these animals both at the gross and microscopic level, showing no sign of mononuclear cell inflammation. In contrast, as shown in Table 5, all 10 of the Ovcar-5 and all 5 of the Skov3 animals injected with 40 million tumor cells infected with the backbone virus Ad-Lp-LacZ had remarkable sign of tumor cells, either at the gross level or at the microscopic level. This data shows that the toxic effect of the vector/5FC system is specific for the tumor cells, and that there is no bystander effect damage on the normal cells of the peritoneum.

Table 5. Tumor Growth In Animals Injected with Adenoviral LP Vectors

	Ad-Lp-LacZ infected	Ad-Lp-CD infected
Ovcar-5 (100MOI)	10/10 (100%)	0/10 (0%)
Skov3 (80MOI)	5/5 (100%)	0/5 (0%)

The SCID mice were injected with 40 million Ovcar-5 or Skov3 tumor cells which were infected with Ad-Lp-LacZ viral vector or Ad-Lp-CD viral vector. From the second day 500mg/Kg 5-FC was injected each day for 10 days. Animals were autopsied at 21

days after tumor cell injection and the tumor nodules in the peritoneal cavity were measured.

Example 11

Preparation of conditionally replication competent adenovirus vectors.

To produce a conditionally replication competent adenovirus vector an adenoviral vector was assembled with the tumor specific L-plastin promoter controlling the adenoviral E1A gene which the virus needs to replicate within cells. These vectors are designed to replicate within the tumor cells but not the normal cells, and therefore be directly toxic to the cancer cells, such as ovarian cancer cells, without damaging the normal cells which line the cavity of the body in which the ovarian cancer cells grow. In the assembly strategy employed, the method of homologous recombination in bacterial cells which has recently reported by He *et al.* (1998) was used. A shuttle vector was constructed which contains the left hand inverted terminal repeat, and packaging signal nucleotides 1-480, and 512-5798 of Ad5, the L-plastin/E1A transcription unit, and Ad5 nucleotides 34,931-35,935, which mediate homologous recombination with the larger adenoviral backbone vectors (pAdEasy-1). The ITR and packaging signal sequences are needed for generation or production of the vector in mammalian producer cells. The Ad nucleotides 512-5798 and 34,931-35,935 are needed for homologous recombination with the larger vector. This shuttle vector also contains a kanamycin resistance sequence and an origin of replication of the PBR322.

The addition of vectors in which the L-plastin promoter controls the E1 viral replication genes to cultures of ovarian cancer cell lines results in the death of these cells as compared to a similar culture not exposed to the L-plastin E1 vector. The same vectors do not cause the CCD minimal deviation cell line, which resembles the normal peritoneal lining cells, to round up any more than cultures not exposed to the L-plastin E1 vector. These data indicate that the L-plastin E1 adenoviral vectors which replicate in tumor cells but not in normal cells, and express their therapeutic transgenes in tumor cells but not in normal cells, are useful in the killing of ovarian cancer cells in human patients. In addition, these data indicate that the vectors will not be toxic to the normal cells of the patients.

Example 12

Assembly of the Ad.Lp.E1A/CD adenoviral vector in which the cytosine deaminase is attached to the E1 gene, and both genes are driven by the L -Plastin promoter.

The following assembly strategy utilizes the method of homologous recombination in bacterial cells of He *et al.* (1998). In this method, a shuttle vector is constructed which contains the left hand inverted terminal repeat, and packaging signal nucleotides 1-480, and 512-5798 of Ad5, the L-plastin-E1/CD transcription unit, and Ad5 nucleotides 34,931-35,935, which mediate homologous recombination with the larger adenoviral backbone vectors (pAdEasy-1).

The steps are as follows:

-PCR synthesis of a 5.2 Kb fragment on Ad5DNA as template, which includes nucleotides 512-5798 (E1 and E2 genes). This fragment has P*me* I/*Sal* I sites at either end for directional cloning.

-Cloning of the E1 PCR fragment into the pCRII vector, using the TA Cloning Kit for PCR fragments. Attachment of the IRES/CD gene to the E1A.
Pme I/Sal I double digest of E1/CD PCR product. P*me* I/*Sal* I double digest of the pShuttle vector from He *et al.* (1998).

-Ligation reaction between the *Sal* I/*Pme* I digested large fragment of the pShuttle and the *Sal* I/*Pme* I fragment containing E1, producing pAD shuttle vector.

-Digest pAD shuttle vector with *Not* I (nt 492) and *Sal* I (nt 526). Then, insert with directional cloning the 2.4 Kb L-Plastin promoter shown by Chung *et al.* (1999) to produce tumor specific expression of heterologous reporter genes. Also construct another shuttle with the CMV promoter as a *Not* I/*Sal*I fragment at the site of the E1 promoter. This shuttle vector is designated pADCMVE1A/CD.

-Digest the pad shuttle with *Me* I. This produces an open linear form of DNA suitable for homologous recombination with pAdEasy-1 which contains the rest of the 35 Kb of adenoviral sequences.

-Electroporate a 10/1 ratio mixture of the *Pme* I digested open linear form of the pADE12.4LPE1A/CD shuttle vector to the supercoiled form of pAdEasy-1 of 10/1 into the BJ5182 *E. coli* competent cell host strain. Those clones grown up in kanamycin, which

appeared to be recombinant, then were electroplated into the DH10B Rec A positive bacterial host strain.

-The plasmid DNA is isolated and analyzed by restriction endonuclease digestion. Those clones shown to be recombinant, are then transfected into the 293 cells after *Pac I* digestion, and the virus isolated from the lysate after one week's amplification. Following titering, the viral particles are ready for purification by cesium chloride density gradient ultracentrifugation.

-The Lp-E1A transcription unit is attached to a CD gene by a IRES linker to produce a bi-cistronic transcription unit under the control of the L-plastin promoter.

Example 13

Purging of autologous hematopoietic cell samples.

To evaluate the ability of the vectors of the invention to purge bone marrow samples of neoplastic cells, a mixture of $2x10^6$ MCF-7 cells (a breast cancer cell line) in a 100-fold excess of HL60 cells was exposed to the CD adenoviral vector (400MOI) for 90 minutes; the cells were then subjected to 10-fold serial dilutions and plated overnight. The nonadherent cells were rinsed away, and the cells were incubated in 500 μmol/L 5-FC for 14 days. In a second experiment, we made a mixture of $9x10^7$ MCF-7 cells and $7x10^9$ HL60 hematopietic cells, exposed the cells to the cytosine deaminase vector (200MOI) for 90 minutes, plated the cells directly or after serial dilution by factors of 10 up to 1/1,000,000 and the incubated overnight, rinsed off the nonadherent cells and incubated the cells for 14 days in 500μMol/L 5-FC. Colony counts at the end of this period, even in the flask that were inoculated with 10^7 NMCF-7 cells, showed no colonies detectable even in the undiluted cultures, in which the cells have been exposed to the cytosine deaminase vector and incubated in 5-FC. See Table 6.

To test the effect of the 5-FC/CD adenoviral system on the viability of the more primitive hematopoietic cell, marrow cells collected from 5-FU-treated male donor mice were exposed to the cytosine deaminase adenoviral vector for 90 minutes. We then incubated the marrow cells for 4 days in serum-free medium (QBSF 58 medium) supplemented with 100 ng/mL stem cell factor and 500 μmol/L 5-FC. One to two million of these cells were then used in the transplant into lethally irradiated female mice. Sixty-

eight percent of the mice survived the transplant. As shown in Fig. 7, most of the deaths occurred before day 10 after transplantation are usually ascribed to infection or radiation toxicity, rather than to failure to engraft. Thus, most of the hematopoietic reconstituting cells survived the *in vitro* incubation with 5-FC after exposure to the cytosine deaminase adenoviral vector.

Because of earlier data indicating a 1 million fold reduction of the BCC in an excess of hematopoietic cells was achieved by incubation of the cytosine deaminase vector exposed cells to 10 or more days of 5-FC incubation (see Table 6), we also used the mouse model to test whether CD adenoviral-infected cells, exposed to 14 days of 5-FC immediately after infusion, would maintain the reconstituting capability. One to two million of these cells were used in the transplant into lethally irradiated female recipient mice. As shown by the data in Table 7, the survival of the mice undergoing transplantation with vector-exposed cells with or without the posttransplantation 14-day infusion of 5-FC, at intraperitoneal doses of 5-FC that generate 500 μmol/L peak serum concentrations, was not significantly different from the survival of mice undergoing transplantation with vector-exposed cells without 5-FC treatment (Table 7).

Individual posttransplantation white blood cell counts (WBC) and differential counts performed in all animals in each group (transplants with cells exposed to vector followed by 4 days of *in vitro* 5-FC exposure) showed no differences in leukocytes, lymphocytes, platelets, or hematocrit volumes among the different transplant groups when determined at 2 or 6 months after transplantation (data not shown). These data suggest that the grafts are stable in all of the treatment groups.

To measure specifically whether the male donor cells that were exposed before transplantation *in vitro* to the cytosine deaminase adenoviral vector followed by 4 days of pretransplantation *in vitro* exposure to 5-FC or exposed to the vector before transplantation followed by 14 days of posttransplantation 5-FC exposure could stably repopulate the female recipient lethally irradiated mice, lethally irradiated female recipient mice transplanted with Ad.CMV-CD-exposed cells exposed to 5-FC either before or after transplantation were killed 1 to 6 months after transplantation. Then, G-banding cytogenetic analysis for the Y chromosome was performed on the bone marrow cells from these animals. We performed this analysis both for the animals transplanted with vector-

exposed cells that either were incubated in vitro for 4 days in QBSF58 serum-free medium supplemented with 500 μmol/L 5-FC (2 animals at 1 to 2 months after transplantation) or were exposed after transplantation in vivo to 5-FC for 14 days (4 animals at 6 months after transplantation).

Because all transplants involved the infusion of male donor cells into lethally irradiated female recipients, we were able to use cytogenetics to test stable and complete engraftment of the Ad.CMV-CD and 5-FC-treated cells. All of the metaphase spreads studied for each of 2 fecal recipient animals 1 and 2 months after transplantation showed 100% of the cells as donor male cells. In addition, greater than 99% of the metaphases studied at 6 months in both groups (4 days of in vitro 5-FC exposure in QBSF58 serum-free medium before transplantation or 14 days of posttransplantation 5-FC in vivo exposure) showed a male karyotype. These results demonstrate that the engrafting capability of the male reconstituting hematopoietic cells was not affected by the pretransplantation Ad.CMV-CD exposure followed by either 4 days of pretransplantation 5-FC in vitro or 14 days of posttransplantation in vivo 5-FC administration. All of these data show that a sufficient number of the hematopoietic reconstituting cells survived the 5-FC/adenoviral vector exposure to generate complete and stable engraftment in lethally irradiated female recipients.

Table 6. MCF-7 Clonogenic Cells After Exposure of a Mixture of 9×10^7 MCF-7 Cells in a 100-Fold Excess of HL60 Cells to the CD Adenoviral Vector (200 MOI) FOR 90 Minutes

MCF-7 Cells Plated at Start of Experiment	Colonies Formed at 10-14 Days of 5-FC Incubation			
	5-FC/CDAV	5-FC Alone	CDAV Alone	No 5-FC No CDAV
90 million	None	TMTC	TMTC	TMTC
10 million	None	TMTC	TMTC	TMTC
2 million	None	1,694	2,800	2,100
200,000	None	484	520	600
20,000	None	138	111	146
2,000	None	34	23	33
200	None	12	0	4
20	None	2	0	4

Serial 10-fold dilutions of the cell were followed by plating over-night and rinsing off of the non-adherent cells. The adherent cells were then incubated in 500 μmol/L 5-FC for 10 to 14 days. The colonies were then counted. One colony equals 20 cells.

Abbreviations: TMTC, too many to count; CDAV, cytosine deaminase adenoviral vector.

Table 7. Transplantation of Marrow Cells Exposed Before Transplantation to Cytosine deaminase Adenoviral Vector Followed by Posttransplantation in Vivo Exposure to 14 Days of 5-FC

	Animal Survival Posttransplantation
XRT alone	32% (8/25)
XRT/FBM	80% (20/25)
XRT/FBM/5-FC	74% (11/15)
XRT/FBM/5-FC/AD	72% (18/25)

Results of engraftment (survival) after transplantation with vector-exposed cells followed by 14 days of posttransplant 5-FC (each dose is sufficient to generate a 2-hour peak level of 500 μmol/L).

Abbreviations: XRT, total body irradiation; FBM, fresh bone marrow without incubation; AD, CD adenoviral vector.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

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